

# Elucidating the Role of Macrophages in Visceral Leishmaniasis

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## Introduction

Leishmaniasis are a collection of human diseases caused by protozoan parasite species that belong to the genus *Leishmania*. Parasites are transmitted by the bite of infected female sandflies. Humans are secondary targets of infection, with rodents, sloths, and dogs being the primary reservoirs of parasites that are infectious to man (Liew *et al.* 1993). Currently there is no vaccine available, and leishmaniasis are endemic in 88 countries, with 350 million people at risk. An estimated 12 million people are infected worldwide, with 1.5 – 2 million new cases each year. The three main clinical manifestations are cutaneous, mucocutaneous, and visceral disease. Cutaneous disease manifests as skin ulcerations that typically heal spontaneously. Mucocutaneous disease afflicts (peri)-oral and (peri)-nasal mucosal tissue, resulting in severe disfigurement due to extensive tissue destruction. This study focuses on visceral leishmaniasis (VL), which is caused by *Leishmania donovani*, *L. chagasi*, and *L. infantum*. There are approximately 500,000 new cases of VL annually, with over 90% of the cases occurring in Bangladesh, Nepal, India, Brazil, and Sudan (WHO Factsheet). VL causes profound hepatosplenomegaly due to widespread dissemination of parasites throughout the reticuloendothelial system. Other symptoms include fever, malaise, weight loss, and anemia (Liew *et al.* 1993, Roberts *et al.* 2000). Visceral disease is fatal without chemotherapeutic intervention, and is sometimes fatal even with treatment (Roberts *et al.* 1993). Current drug

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treatment options include antimonials, amphotericin B, and miltefosine. However, none of these drugs is an ideal treatment option due to high costs, adverse side effects, and emerging drug resistance (Murray *et al.* 2005).

*Leishmania* species are particularly challenging to treat, because they parasitize host macrophages – phagocytic cells that are normally responsible for the uptake and clearance of invading microorganisms. Parasites actively replicate in parasitophorous vacuoles within the macrophage, and evade immune detection by dampening normal macrophage antimicrobial responses. Parasites are able to alter the antigen presenting functions and MHC class II expression of macrophages, both of which are necessary for effective T-cell communication and activation. Macrophage-T-cell interactions are key because the activation of certain T-cell subsets leads to the production of inflammatory cytokines that can activate macrophages to effectively kill parasites (Alexander and Russell 1992). Early immunological studies in mice infected with *L. major*, a parasite that causes self-limiting cutaneous disease, revealed that type 1 T helper cell (Th1) responses involving the production of cytokines such as IFN- $\gamma$ , IL-12 and TNF- $\alpha$  by immune cells results in the killing of parasites within macrophages and resolution of infection, whereas type 2 T helper cell (Th2) responses involving the production of IL-4, IL-13 and IL-10 by immune cells is associated with disease exacerbation and non-healing phenotypes (Alexander and Bryson 2005). However, the role of Th1/Th2 type responses is not as straightforward in VL, as IL-4 has been shown to be necessary for the success of certain drug treatments (Alexander *et al.* 2000), even though inhibition of IL-10 is protective (Murray *et al.* 2002). Nonetheless, the development of a strong Th1 response with the production of IFN- $\gamma$  and IL-12 is critical for parasite clearance (Taylor and Murray 1997, Murray and Delph-Etienne

2000, Murray *et al.* 2006). In fact, granuloma formation in the liver, which is required for resolution of disease, is IFN- $\gamma$  dependent (Murray and Delph-Etienne 2000).

## **Materials and Methods**

**Mice.** Eight to twelve week old BALB/c wild-type (WT) mice purchased from Harlan laboratories (Indianapolis, IN) were used for all experiments.

**Parasite.** Sudan strain *L. donovani* was harvested from the spleens of sick Golden Syrian hamsters. Mice were infected with 10 million amastigotes in a volume of 100  $\mu$ l intravenously (i.v.) via tail vein injection.

**Clodronate.** Liposomal clodronate was obtained from Dr. Van Rooijen ([www.clodronateliposomes.org](http://www.clodronateliposomes.org)). Mice were initially injected with 200  $\mu$ l i.v. via the tail vein and 400  $\mu$ l intraperitoneally (i.p.). For timepoints beyond 7 days post drug treatment mice were re-treated 7 days after the initial treatment with 100  $\mu$ l i.v. and 200  $\mu$ l i.p. Controls received PBS containing liposomes.

**Parasite Burdens.** Impression smears of spleens and livers harvested from drug-treated and control mice were made on glass slides. These slides were then stained with Giemsa. Parasites and nucleated host cells were counted. Leishman-Donovan units (LDUs) were then calculated as the number of parasites per 1000 nucleated cells to express parasite levels.

**ELISA.** Splenocytes harvested from drug-treated and control mice were plated at a concentration of 0.5 million cells per well in triplicate in 96 well plates. Cells were then stimulated with 20  $\mu$ g/ml of soluble *L. donovani* antigen obtained from repeated freeze thaw cycles. Cell supernatants were collected 72 hours after stimulation and levels of secreted IFN- $\gamma$ , IL-12, IL-4, and IL-10 were then measured using an enzyme-linked immunosorbent assay (ELISA).

**Histopathology.** Tissue sections from the livers and spleens of drug-treated and control mice were collected and sent for histological processing at The Ohio State University Core Pathology Facility. Tissues were cut into 6 micron sections and stained with hematoxylin and eosin.

The analysis performed at each timepoint is shown in Figure 1.

## **Results and Discussion**

**Preliminary Data.** Recently, our laboratory found that STAT-1 and T-bet, transcription factors which both play a key role in IFN- $\gamma$  signaling and production by CD4<sup>+</sup> Th1 cells (fig. 2), unexpectedly play distinct roles in the outcome of VL. Both T-bet and STAT-1 knock-out (KO) mice on a BABL/c background were unable to mount Th1 responses, as evidenced by cytokine ELISA data. However, liver and spleen parasite burdens showed that STAT-1 KO mice were unexpectedly resistant to infection, while T-bet mice were highly susceptible. Further analysis revealed that STAT-1 KO mice have severe impairments in both T-cell (data not shown) and macrophage trafficking (fig. 3) to the liver following infection (Rosas *et al.* 2006). Since Rag2 KO mice, which lack T-cells, are highly susceptible to *L. donovani* infection, we hypothesize that the resistant phenotype seen in STAT-1 KO mice is due to the reduction in macrophage, and not T-cell, trafficking to the liver. Clodronate containing liposomes have been shown to deplete macrophages via apoptosis, particularly splenic macrophages and Kupffer cells – resident liver macrophages (Van Rooijen 1989). Clodronate (fig. 4) is a bisphosphonate drug used to inhibit osteoclast activity in patients with osteolytic bone diseases ([www.clodronateliposomes.org](http://www.clodronateliposomes.org)). To determine the role of macrophages in *L. donovani* infection, wild-type (WT) BALB/c mice were treated clodronate-containing liposomes (control mice were treated with PBS containing liposomes) both pre- and post-*L. donovani* infection.



**Clodronate treated mice had markedly reduced parasite burdens.** Mice treated with clodronate liposomes both 2 days prior to infection (fig 5a) or 1 (fig 5b) or 6 days (fig 5c) post-infection had significantly reduced parasite burdens in both their livers (fig 5) and spleens (data not shown) compared to PBS controls. The presented data indicate that macrophage depletion both early and late in infection is capable of reducing parasite burdens in the tissues affected by *L. donovani*. These effects most likely result from the elimination of actively infected macrophages and the elimination of monocytes that could potentially serve as future reservoirs of infection. Without the presence of host cell macrophages to parasitize, *L. donovani* die off within their hosts. It is important to note, however, that the macrophage depletion afforded by clodronate treatment is transient, and repopulation of blood monocytes in mice begins within eight days.

**Clodronate treatment appears to dampen immune responses.** Stimulated immune cells isolated from the spleens of mice treated with clodronate 2 days prior to infection had reduced levels of IFN- $\gamma$ , IL-12, IL-4, and IL-10 as measured by ELISA (fig 6a). A similar trend was seen in mice treated with clodronate 1 day (data not shown – trend is the same as seen in fig 6b) and 6 days (fig 6b) post-infection, with the exception of elevated IL-4. This data indicates that macrophage depletion dampens the overall immune response following infection, with the exception of IL-4 production in mice treated post-infection. This reduction of IFN- $\gamma$  is not surprising, because the production of IFN- $\gamma$  by T cells is dependent on their activation via macrophage communication. Additionally, IL-12 and IL-10 are cytokines mainly secreted by macrophages, so following depletion one would expect to see reduced levels. IL-4 is a cytokine produced exclusively by Th2 CD4<sup>+</sup> T-cells. It is probably reduced in mice treated with clodronate prior to infection because no macrophages are ever infected, and therefore no immune

response is ever initiated. It is probably seen elevated in mice treated post-infection, however, because the initial macrophage infection is capable of stimulating T-cell differentiation. However, without a sustained macrophage signal (the signal is lost due to clodronate depletion) the default program of differentiating T-cells is into the Th2 subset, which secretes IL-4.

**Early clodronate treatment reduces liver immunopathology.** Mice treated with clodronate 2 days prior (data not shown – similar to fig 7a) to infection or 1 day post infection (fig 7a) showed little or no immunopathology when compared to PBS treated controls which exhibited diffuse foci of inflammation and granuloma formation. However, mice treated with clodronate 6 days post-infection showed immunopathology similar to PBS controls (fig 7b), despite their low parasite load (fig 5c). These data indicate that while BALB/c WT mice have established infection and developed immunopathology by day 6 in their livers, clodronate treatment is still effective in eliminating parasites following the induction of macrophage apoptosis.

## **Implications**

This research may aid in the initial development of new drug treatment options for visceral leishmaniasis. Many of the current treatment options are very expensive and require long hospital stays because they must be administered intravenously over time. While miltefosine is administered orally, patient noncompliance could lead to the development of resistance against this drug. Clodronate, or other drugs that induce macrophage apoptosis, could ideally be administered as 1-2 injections in a clinical setting, eliminating the need for hospital stays. Also, due to clodronate's mechanism of action, patient noncompliance and drug resistance would not be an issue. Additionally, other diseases in which pathogens reside and multiply within host cell macrophages, such as tuberculosis, could benefit from the development of a drug that selectively and temporarily depletes host cell macrophages.

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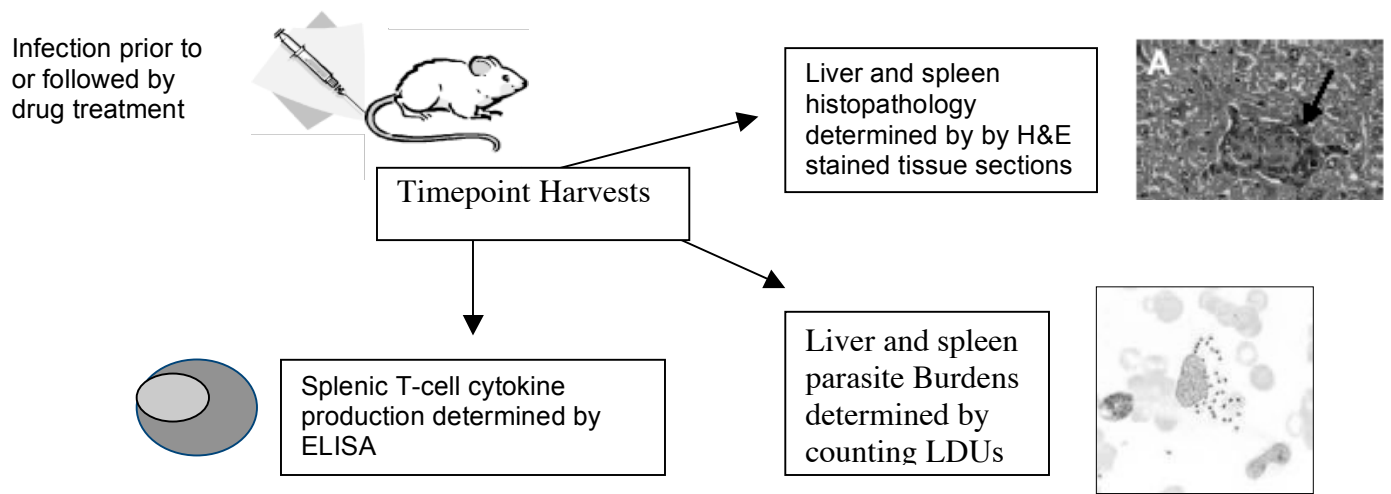


Figure 1. General scheme of timepoint analysis.

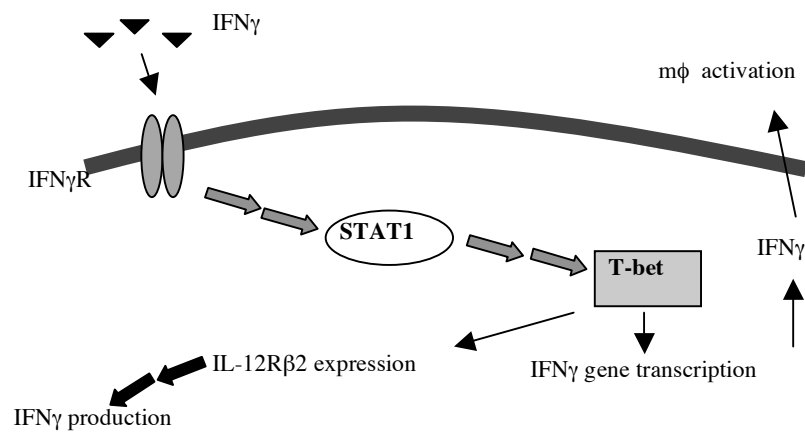


Figure 2. Diagram of IFN-  $\gamma$  signaling in CD4+ T cells

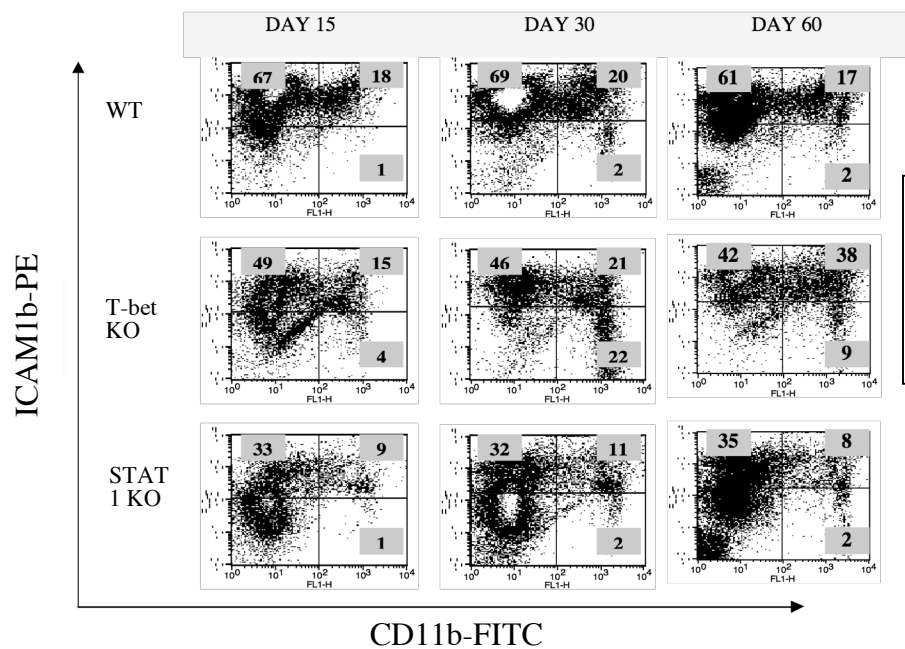


Figure 3. Flow cytometry data of *L. donovani* infected mouse liver tissue. Macrophages stain double positive for the markers ICAM-1b and CD11b.

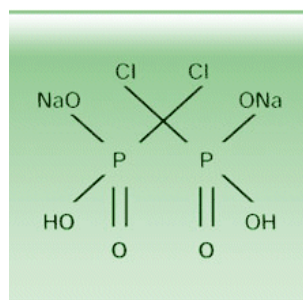


Figure 4. Clodronate Structure

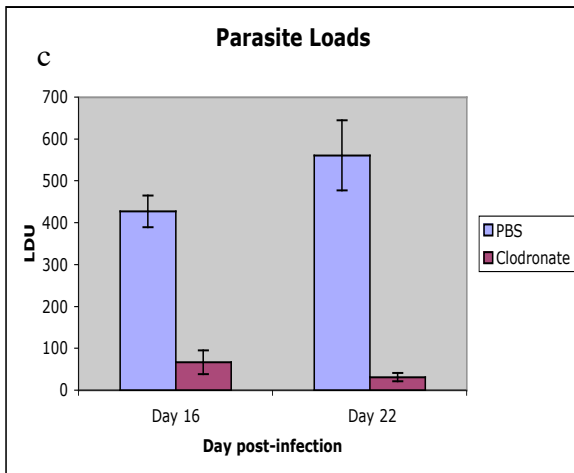
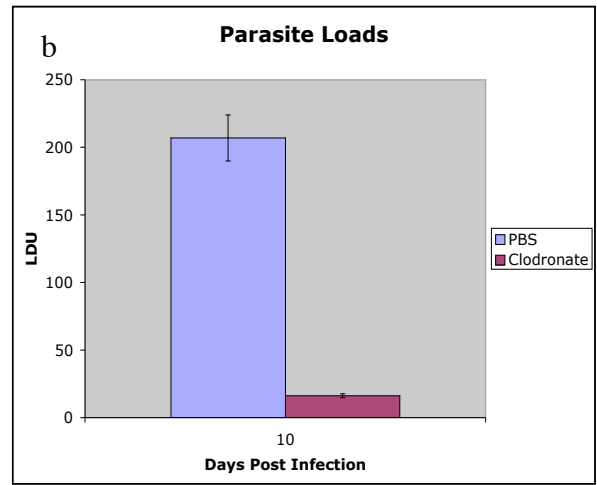
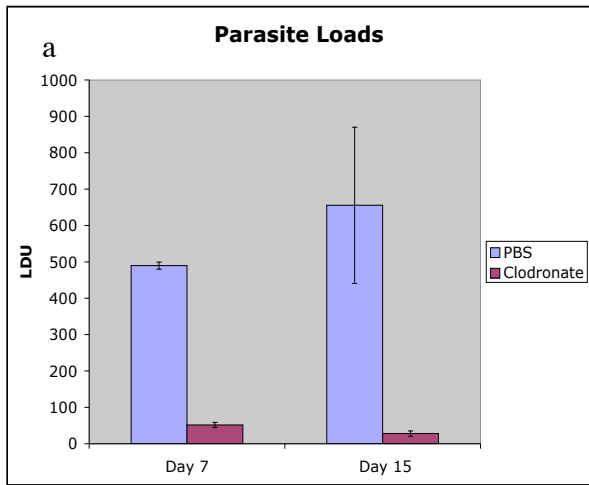


Figure 5. Parasite burdens in the liver of mice treated with clodronate 2 days prior to infection (a), 1 day (b), or 6 days post-infection. Data are represented as LDUs. Each bar represents the mean of data obtained from 3-4 mice. Error bars represent standard error.

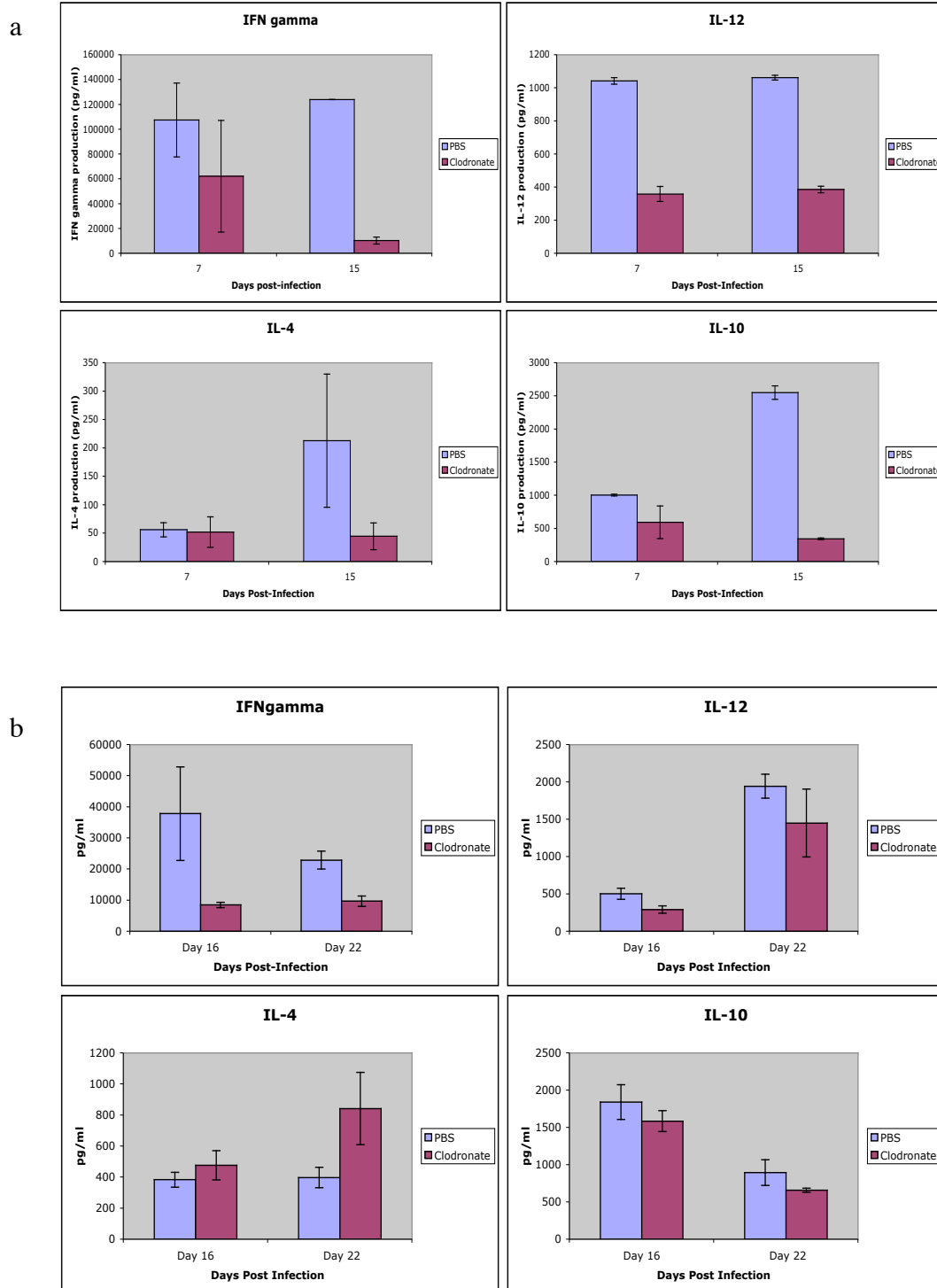


Figure 6. Cytokine production from the supernatants of stimulated splenocytes harvested from mice treated 2 days prior to infection (a) or 6 days post infection (b) with clodronate, measured using ELISA. Data represent the means generated from triplicate samples of 3-4 mice per group. Error bars indicate standard error.



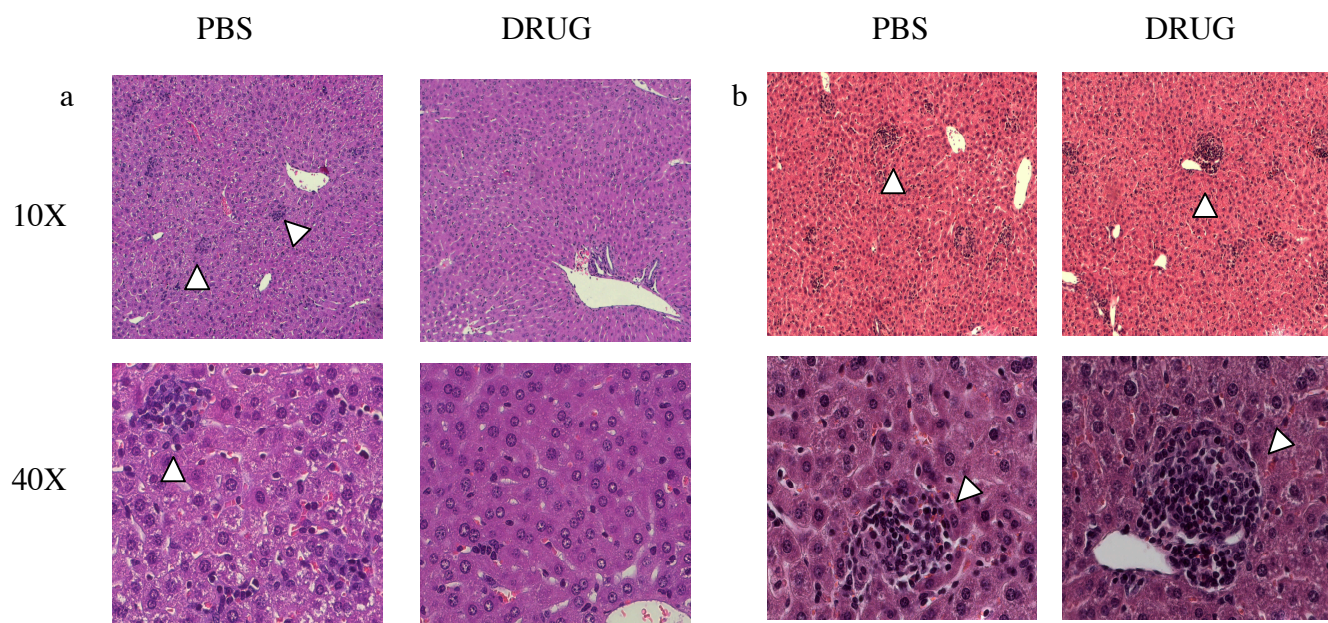


Figure 7. Histopathology from mice treated with clodronate 1 day (a) or 6 days (b) after infection. Arrowheads indicate granulomas.